TRANSMITTAL LETTER TO THE UNITED STATES ATTORNEY'S DOCKET NUMBER 50061

DESIGNATED/ELECTED OFFICE (DO/EO/US)
CONCERNING A FILING UNDER 35 U.S.C. 371 U.S. APPLICATION NO. (If known, see 37 CFR 1.5)

INTERNATIONAL APPLICATION NO.	INTERNATIONAL FILING DATE	PRIORITY DATE CLAIMED
PCT/EP00/05258	**7 June 2000**	**17 June 1999**

TITLE OF INVENTION: METHOD OF GENERATING PLANTS WITH AN INCREASED CONTENT OF FLAVONOIDS AND PHENOLIC CONSTITUENTS

APPLICANT(S) FOR DO/EO/US Wilhelm RADEMACHER, Klaus KRAEMER, Juergen SCHWEDEN

Applicant herewith submits to the United States Designated/Elected Office (DO/EO/US) the following items and other information:

1. /X/ This is a FIRST submission of items concerning a filing under 35 U.S.C. 371.

2. / / This is a SECOND or SUBSEQUENT submission of items concerning a filing under 35 U.S.C. 371.

3. /X/ This express request to begin national examination procedures (35 U.S.C.371(f)) at any time rather than delay examination until the expiration of the applicable time limit set in 35 U.S.C. 371(b) and PCT Articles 22 and 39(1).

4. /x/ A proper Demand for International Preliminary Examination was made by the 19th month from the earliest claimed priority date.

5. /X/ A copy of the International Application as filed (35 U.S.C. 371(c)(2)).

   a./X/ is transmitted herewith (required only if not transmitted by the International Bureau).
   b./ / has been transmitted by the International Bureau.
   c./ / is not required, as the application was filed in the United States Receiving Office (RO/US0).

6. /X/ A translation of the International Application into English (35 U.S.C. 371(c)(2)).

7. / / Amendments to the claims of the International Application under PCT Article 19 (35 U.S.C. 371(c)(3)).

   a./ / are transmitted herewith (required only if not transmitted by the International Bureau).
   b./ / have been transmitted by the International Bureau.
   c./ / have not been made; however, the time limit for making such amendments has NOT expired.
   d./ / have not been made and will not be made.

8. / / A translation of the amendments to the claims under PCT Article 19(35 U.S.C. 371(c)(3)).

9. /X / An oath or declaration of the inventor(s)(35 U.S.C. 171(c)(4)).

10./ / A translation of the annexes to the International Preliminary Examination Report under PCT Article 36 (35 U.S.C. 371(c)(5)).

Items 11. to 16. below concern other document(s) or information included:

11./ / An Information Disclosure Statement under 37 CFR 1.97 and 1.98.

12./X / An assignment document for recording. A separate cover sheet in compliance with 37 CFR 3.28 and 3.31 is included.

13./ x / A FIRST preliminary amendment.
/ / A SECOND or SUBSEQUENT preliminary amendment.

14./ / A substitute specification.

15./ / A change of power of attorney and/or address letter.

16./x / Other items or information.
International Search Report
International Preliminary Examination Report

U.S. Appln. No. (If Known)	INTERNATIONAL APPLN. NO.	ATTORNEY'S DOCKET NO.
	PCT/EP00/05258	50061

17. /X/ The following fees are submitted	CALCULATIONS	PTO USE ONLY
BASIC NATIONAL FEE (37 CFR 1.492(a)(1)-(5)): Search Report has been prepared by the EPO or JPO.....$890.00	**890.00**	
International preliminary examination fee paid to USPTO (37 CFR 1.482).....$710.00		
No international preliminary examination fee paid to USPTO (37 CFR 1.482) but international search fee paid to USPTO (37 CFR 1.445(a)(2)).....$740.00		
Neither international preliminary examination fee (37 CFR 1.482) nor international search fee (37 CFR 1.445(a)(2)) paid to USPTO .....$ 1,040.00		
International preliminary examination fee paid to USPTO (37 CFR 1.482) and all claims satisfied pro -visions of PCT Article 33(2)-(4).....$100.00		
ENTER APPROPRIATE BASIC FEE AMOUNT =	$ 890.00	
Surcharge of $130.00 for furnishing the oath or declaration later than / / 20 / /30 months from the earliest claimed priority date (37 CFR 1.492(e)).		

Claims	Number Filed	Number Extra	Rate	
Total Claims	6	-20	X$18.	
Indep.Claims	1	-3	X$84.	
Multiple dependent claim(s)(if applicable)			+280.	
TOTAL OF ABOVE CALCULATION			= **890.**	
Reduction of 1/2 for filing by small entity, if applicable. Verified Small Entity statement must also be filed (Note 37 CFR 1.9, 1.27, 1.28).				
SUBTOTAL			= 890.	
Processing fee of $130. for furnishing the English translation later than / /20 / /30 months from the earliest claimed priority date (37 CFR 1.492(f)).		+		
TOTAL NATIONAL FEE			= **890 .**	
Fee for recording the enclosed assignment (37 CFR 1.21(h)). The assignment must be accompanied by an appropriate cover sheet (37 CFR 3.28, 3.31) $40.00 per property		=	40.	
TOTAL FEES ENCLOSED			= **$ 930.00**	

Amount to be refunded: $ _____

Charged $ _____

a./X/ A check in the amount of $ 930.00 to cover the above fees is enclosed.

b./ / Please charge my Deposit Account No._____in the amount of $_____ to cover the above fees. A duplicate copy of this sheet is enclosed.

c./X/ The Commissioner is hereby authorized to charge any additional fees which may be required, or credit any overpayment to Deposit Account No. **11-0345**. A duplicate copy of this sheetis enclosed.

NOTE: Where an appropriate time limit under 37 CFR 1.494 or 1.495 has not been met, a petition to revive (37 CFR 1.137(a) or (b) must be filed and granted to restore the application to pending status.

Herbert B Keil
SIGNATURE

SEND ALL CORRESPONDENCE TO:
KEIL & WEINKAUF
1101 Connecticut Ave., N.W.
Washington, D. C. 20036

Herbert B. Keil
NAME

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re the Application of)
RADEMACHER et al.) BOX PCT
)
International Application)
PCT/EP 00/05258)
)
Filed: June 7, 2000)
)

For: METHOD OF GENERATING PLANTS WITH AN INCREASED CONTENT OF FLAVONOIDS AND PHENOLIC CONSTITUENTS

PRELIMINARY AMENDMENT

Honorable Commissioner of
Patents and Trademarks
Washington, D.C. 20231

Sir:

Prior to examination, kindly amend the above-identified application as follows:

IN THE CLAIMS

Kindly amend the claims as shown on the attached sheets.

REMARKS

The claims were amended in the preliminary examination. The claims have been amended further to eliminate multiple dependency and to place them in better form for U.S. filing. No new matter is included.

A clean copy of the claims is attached.

Favorable action is solicited.

Respectfully submitted,

KEIL & WEINKAUF

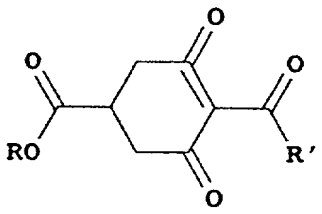
Herbert B. Keil
Reg. No. 18,967

1101 Connecticut Ave., N.W.
Washington, D.C. 20036

(202)659-0100

CLEAN VERSION OF AMENDED CLAIMS - **OZ 0050/50061**

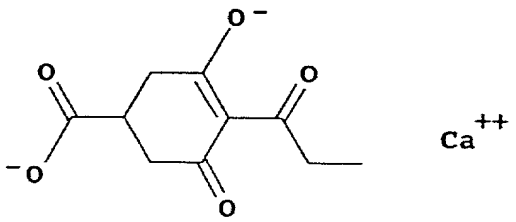
1. A method of increasing and qualitatively modifying the content of flavonoids and phenolic constituents in grapevines, cherries, plums, sloes, blueberries, strawberries, citrus fruit, pawpaw, red cabbabe, broccoli, Brussels sprouts, kale, carrots, parsley, celery/celeriac, onions, garlic, tea, coffee, cacao, maté, hops, soya, oilseed rape, oats, wheat, rye, *Aronia melanocarpa* or *Ginkgo biloba*, which comprises treating the plants with an acylcyclohexadione of the formula I



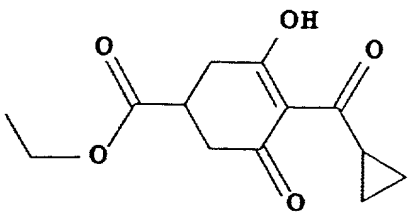
I,

where R is hydrogen or C_1-C_6-alkyl and R' is C_1 -C_6-alkyl or C_3-C_6-cycloalkyl, or with a suitable salt of I.

2. A method as claimed in claim 1, wherein the plants are treated with an acylcyclohexadione of the formula II and/or the formula III



Ca^{++} II



III.

3. A method as claimed in claim 1, wherein the content of flavonoids and phenolic constituents of grapevines is increased and qualitatively modified.

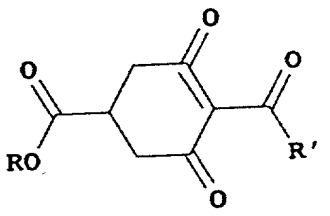
4. A method as claimed in claim 1, wherein the content of flavonoids with an unsubstituted C atom in the 3-position, and of the oligomers and polymers of these flavonoids, is increased.

5. The use of grapevines, cherries, plums, sloes, blueberries, strawberries, citrus fruit, pawpaw, red cabbage, broccoli, Brussels sprouts, kale, carrots, parsley, celery/celeriac, onions, garlic, tea, coffee, cacao, mate, hops, soya, oilseed rape, oats, wheat, rye, *Aronia melanocarpa* or *Ginkgo biloba,* which have been treated with an acylcyclohexadione as set forth in claim 1, of parts of these plants or of products prepared with these plants (juices, teas, extracts, fermentation products and fermentation residues) for the preparation of curative compositions, health-promoting compositions or tonics for humans and animals, and of cosmetics.

6. An extract, juice, wine or press cake with an increased qualtitatively modified content of flavonoids and other phenolic constituents, obtainable from grapes of a red grapevine variety, the grapevine plant previously having been treated with at least one acylcyclohexadione of the formula I, II or III as set forth in claim 1.

MARKED UP VERSION OF AMENDED CLAIMS - **OZ 0050/50061**

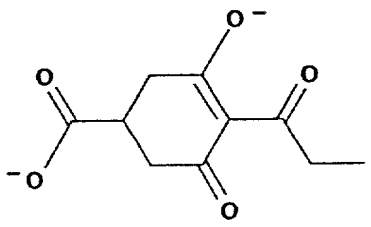
1. A method of increasing and qualitatively modifying the content of flavonoids and phenolic constituents in grapevines, cherries, plums, sloes, blueberries, strawberries, citrus fruit, pawpaw, red cabbabe, broccoli, Brussels sprouts, kale, carrots, parsley, celery/celeriac, onions, garlic, tea, coffee, cacao, maté, hops, soya, oilseed rape, oats, wheat, rye, *Aronia melanocarpa* or *Ginkgo biloba*, which comprises treating the plants with an acylcyclohexadione [[sic]] of the formula I



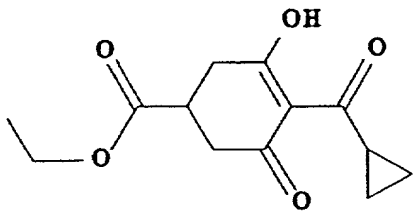
I,

where R is hydrogen or C_1-C_6-alkyl and R' is C_1 -C_6-alkyl or C_3-C_6-cycloalkyl, or with a suitable salt of I.

2. A method as claimed in claim 1, wherein the plants are treated with an acylcyclohexadione [[sic]] of the formula II and/or the formula III



Ca^{++} II



III.

3. A method as claimed in claim 1, wherein the content of flavonoids and phenolic constituents of grapevines is increased and qualitatively modified.

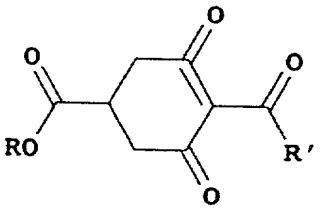
4. A method as claimed in claim 1, wherein the content of flavonoids with an unsubstituted C atom in the 3-position, and of the oligomers and polymers of these flavonoids, is increased.

5. The use of grapevines, cherries, plums, sloes, blueberries, strawberries, citrus fruit, pawpaw, red cabbage, broccoli, Brussels sprouts, kale, carrots, parsley, celery/celeriac, onions, garlic, tea, coffee, cacao, mate, hops, soya, oilseed rape, oats, wheat, rye, *Aronia melanocarpa* or *Ginkgo biloba,* which have been treated with an acylcyclohexadione as set forth in claim 1, of parts of these plants or of products prepared with these plants (juices, teas, extracts, fermentation products and fermentation residues) for the preparation of curative compositions, health-promoting compositions or tonics for humans and animals, and of cosmetics.

6. An extract, juice, wine or press cake with an increased qualtitatively modified content of flavonoids and other phenolic constituents, obtainable from grapes of a red grapevine variety, the grapevine plant previously having been treated with at least one acylcyclohexadione of the formula I, II or III as set forth in claim 1.

CLAIMS AS FILED **- OZ 0050/50061**

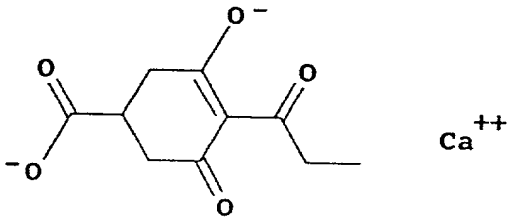
1. A method of increasing and qualitatively modifying the content of flavonoids and phenolic constituents in grapevines, cherries, plums, sloes, blueberries, strawberries, citrus fruit, pawpaw, red cabbabe, broccoli, Brussels sprouts, kale, carrots, parsley, celery/celeriac, onions, garlic, tea, coffee, cacao, maté, hops, soya, oilseed rape, oats, wheat, rye, *Aronia melanocarpa* or *Ginkgo biloba*, which comprises treating the plants with an acylcyclohexadione of the formula I



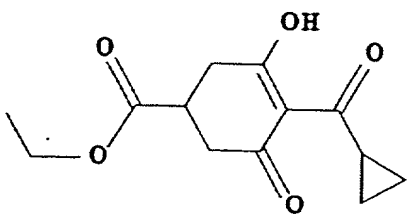
I,

where R is hydrogen or C_1-C_6-alkyl and R' is C_1 -C_6-alkyl or C_3-C_6-cycloalkyl, or with a suitable salt of I.

2. A method as claimed in claim 1, wherein the plants are treated with an acylcyclohexadione of the formula II and/or the formula III



II



III.

CLAIMS AS FILED - OZ 0050/50061

3. A method as claimed in claim 1, wherein the content of flavonoids and phenolic constituents of grapevines is increased and qualitatively modified.

4. A method as claimed in claim 1, wherein the content of flavonoids with an unsubstituted C atom in the 3-position, and of the oligomers and polymers of these flavonoids, is increased.

5. The use of grapevines, cherries, plums, sloes, blueberries, strawberries, citrus fruit, pawpaw, red cabbage, broccoli, Brussels sprouts, kale, carrots, parsley, celery/celeriac, onions, garlic, tea, coffee, cacao, mate, hops, soya, oilseed rape, oats, wheat, rye, *Aronia melanocarpa* or *Ginkgo biloba,* which have been treated with an acylcyclohexadione as set forth in claim 1, of parts of these plants or of products prepared with these plants (juices, teas, extracts, fermentation products and fermentation residues) for the preparation of curative compositions, health-promoting compositions or tonics for humans and animals, and of cosmetics.

6. An extract, juice, wine or press cake with an increased qualtitatively modified content of flavonoids and other phenolic constituents, obtainable from grapes of a red grapevine variety, the grapevine plant previously having been treated with at least one acylcyclohexadione of the formula I, II or III as set forth in claim 1.

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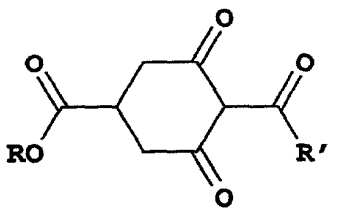
Method of generating plants with an increased content of flavonoids and phenolic constituents.

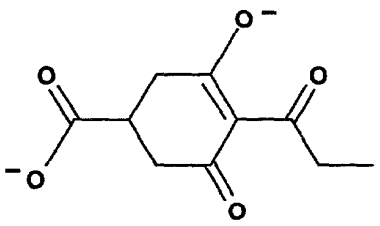
The present invention relates to a method of increasing the content of flavonoids and phenolic constituents in plants, wherein the plants are treated with growth-regulating acylcyclohexanediones of the formula I.



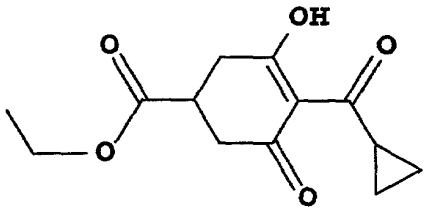
(I)

where R is, in particular, hydrogen, C_1-C_6-alkyl, C_1-C_6-haloalkyl, C_2-C_{10}-alkylthioalkyl or phenyl (substituted or unsubstituted) and R' is hydrogen, C_1-C_6-alkyl, C_3-C_6-cycloalkyl, benzyl (substituted or unsubstituted), phenylethyl, phenoxyethyl, 2-thienylmethyl, alkoxymethyl or alkylthiomethyl, and suitable salts of these compounds.

A method in which the increase is caused by treatment with acylcyclohexanediones such as prohexadione-calcium (II) and/or trinexapac-ethyl (III) is especially preferred.



Ca^{++} (II)



(III)

The invention furthermore relates to the use of plants which have been treated by the method according to the invention with acylcyclohexanediones of the formula I, specifically prohexadione-calcium or with trinexapac-ethyl, or of parts of these plants or of products prepared with them (juices,

infusions, extracts, fermentation products and fermentation residues) for the preparation of curative compositions, health-promoting compositions or tonics for humans and animals, and of cosmetics.

The invention furthermore relates to compositions prepared by the methods according to the invention wherein the grapes of red grapevines are harvested and processed whose anthocyanin production has been prevented fully or partially by treatment with acylcyclohexanediones such as prohexadione-calcium or trinexapac-ethyl and which are therefore distinguished by a qualitatively and quantitatively increased content of flavonoids and other phenolic constituents.

A variety of phenolic substances (phenylpropanoids) are found in plants, for example caffeic acid, ferulic acid, chlorogenic acid, gallic acid, eugenol, lignans, coumarins, lignin, stilbenes (polydatin, resveratrol), flavonoids (flavones, catechines, flavanones, anthocyanidines, isoflavones), polymethoxylated flavones. Accordingly, phenols are also a general component in a large number of plant-derived foodstuffs and stimulants. Certain phenolic substances are of particular importance since, after ingestion together with the food, they may exert an antioxidant effect in the human or animal metabolism (Baum, B. O.; Perun, A. L. Antioxidant efficiency versus structure. *Soc. Plast. Engrs Trans* **2:** 250-257, (1962); Gardner, P.T.; McPhail, D.B.; Duthie, G.G. Electron spin resonance spectroscopic assessment of the antioxidant potential of infusions in aqueous and organic media. *J. Sci. Food Agric.* ***76:*** 257-262, (1997); Rice-Evans, C. A.; Miller, N. J.; Pananga, G. Structure-antioxidant activity relationship of flavonoids and phenolic acids. *Free Radic. Biol. Med.* **20:** 933-956, (1996); Salah, N.; Miller, N. J.; Paganga, G.; Tijburg, L.; Bolwell, G. P.; Rice-Evans, C. Polyphenolic flavonoids as scavenger of aqueous phase radicals and as chain-breaking antioxidants. *Arch Biochem Biophys* **322:** 339-346, (1995); Stryer, L. *Biochemistry* S. Francisco: Freeman, (1975); Vieira, O.; Escargueil-Blanc, I.; Meilhac, O.; Basile, J. P.; Laranjinha, J.; Almeida, L.; Salvayre, R.; Negre-Salvayre, A. Effect of dietary phenolic compounds on apoptosis of human cultured endothelial cells induced by oxidized LDL. *Br J Pharmacol* **123:** 565-573, (1998)). In addition, polyphenols have a multiplicity of effects on the cellular metabolism. Inter alia, signal transduction enzymes such as protein kinase C, tyrosine protein kinase and phosphatidylinositol 3-kinase are modulated (Agullo, G.; Gamet-payrastre, L.; Manenti, S.; Viala, C.; Remesy, C.; Chap, H.; Payrastre, B. Relationship between flavonoid structure and inhibition of phosphatidylinositol 3-kinase: a

comparison with tyrosine kinase and protein kinase C inhibition. *Biochem Pharmacol* **53** :1649-1657, (1997); Ferriola, P. C.; Cody, V.; Middleton, E. Protein kinase C inhibition by plant flavonoids. Kinetic mechanisms and structure activity relationship. *Biochem Pharmacol* **38:** 1617-1624, (1989); Cushman, M.; Nagarathman, D.; Burg, D. L.; Geahlen, R. L. Synthesis and protein-tyrosine kinase inhibitory activity of flavonoids analogues. *J Meed Chem* **34:** 798-806, (1991); Hagiwara, M.; Inoue, S.; Tanaka, T.; Nunoki, K.; Ito, M.; Hidaka, H. Differential effects of flavonoids as inhibitors of tyrosine protein kinases and serine/threonin protein kinases. *Biochem Pharmacol* **37:** 2987-2992, (1988)), which downregulates inducible NO-synthase (Kobuchi, H.; Droy-Lefaix, M. T.; Christen, Y.; Packer, L. *Ginkgo biloba* extract (EGb761): inhibitory effect on nitric oxide production in the macrophage cell line RAW 264.7. *Biochem Pharmacol* **53:** 897-903, (1997)) and which regulates the gene expression of, for example, interleukins and adhesion molecules (ICAM-1, VCAM-1) (Kobuchi, H.; Droy-Lefaix, M. T.; Christen, Y.; Packer, L. *Ginkgo biloba* extract (EGb761): inhibitory effect on nitric oxide production in the macrophage cell line RAW 264.7. *Biochem Pharmacol* **53:**897-903, (1997); Wolle, J.; Hill, R. R.; Ferguson, E.; Devall, L. J.; Trivedi, B. K.; Newton, R. S.; Saxena, U. Selective inhibition of tumor necrosis factor- induced vascular cell adhesion molecule-1 gene expression by a novel flavonoid. Lack of effect on transcriptional factor NF-kB. *Atherioscler Thromb Vasc Biol* **16:** 1501-1508, (1996)). It is proven that these effects have a positive action for preventing cardiovascular diseases, diabetes, various kinds of tumors and other chronic diseases (Bertuglia, S.; Malandrino, S.; Colantuoni, A. Effects of the natural flavonoid delphinidin on diabetic microangiopathy. *Arznei-Forsch/Drug Res* ***45:*** *481-485*, (1995); Griffiths, K.; Adlercreutz, H.; Boyle, P.; Denis, L.; Nicholson, R.I.; Morton, M.S. *Nutrition and Cancer* Oxford: Isis Medical Media, (1996); Hertog, M. G. L.; Fesrens, E. J. M.; Hollman, P. C. K.; Katan, M. B.; Kromhout, D. Dietary antioxidant flavonoids and risk of coronary heart disease: the Zutphen elderly study. *The Lancet* **342:** 1007-1011, (1993); Kapiotis, S.; Hermann, M.; Held, I.; Seelos, C.; Ehringer, H.; Gmeiner, B. M. Genistein, the dietary-derived angiogenesis inhibitor, prevents LDL oxidation and protects endothelial cells from damage by atherogenic LDL. *Arterioscler Thromb Vasc Biol* **17:** 2868-74, (1997); Stampfer, M. J.; Hennekens, C. H.; Manson, J. E.; Colditz, G. A.; Rosner, B.; Willet, W. C. Vitamin E consumption and the risk of coronary disease in women. *New Engl J Med* **328** :1444-1449, (1993); Tijburg, L. B. M.; Mattern, T.; Folts, J. D.; Weisgerber, U. M.; Katan, M. B. Tea flavonoids and cardiovascular diseases: a review. *Crit Rev Food Sci Nutr* **37:** 771-785, (1997);

Kirk, E. A.; Sutherland, P.; Wang, S. A.; Chait, A.; LeBoeuf, R. C. Dietary isoflavones reduce plasma cholesterol and atherosclerosis in C57BL/6 mice but not LDL receptor-deficient mice. *J Nutr* **128:** 954-9, (1998)). A series of curative compositions, health-promoting compositions or tonics whose action is based on their content of phenolic substances is therefore already being obtained from suitable plants (Gerritsen, M. E.; Carley, W. W.; Ranges, G. E.; Shen, C. P.; Phan, S. A.; Ligon, G. F.; Perry, C. A. Flavonoids inhibit cytokine-induced endothelial cell adhesion protein gene expression. *Am J Pathol* **147:** 278-292, (1995); Lin, J. K.; Chen, Y. C.; Huang, Y. T.; Lin-Shiau, S. Y. Suppression of protein kinase C and nuclear oncogene expression as possible molecular mechanisms of cancer chemoprevention by apigenin and curcumin. *J Cell Biochem Suppl* **28-29:**39-48, 1997; Zi, X.; Mukhtar, H.; Agarval, R. Novel cancer chemopreventive effects of a flavonoid antioxidant silymarin: inhibition of mRNA expression of an endogenous tumor promoter TNF alpha. *Biochem Biophys Res Comm* **239:**334-339, 1997). It is also known that certain plant-derived foodstuffs or stimulants prepared from them have a positive effect on various diseases. Resveratrol, which occurs in white wine, but in particular in red wine (in addition to other components), for example, is active against cardiovascular diseases and cancer (Gehm, B.D.; McAndrews, J.M.; Chien, P.-Y.; Jameson, J.L. Resveratrol, a polyphenolic compound found in grapes and wine, is an agonist for estrogen receptor. *Proc Natl Acad Sci USA* ***94***: 14138-14143, (1997); Jang, M.; Cai, L.; Udeani, G.O.; Slowing, K.V.; Thomas, C.F.; Beecher, C.W.W.; Fong, H.H.S; Farnsworth, N.R.; Kinghorn, A.D.; Mehtha, R.G.; Moon, R.C., Pezzuto, J.M. Cancer chemopreventive activity of resveratrol, a natural product derived from grapes. *Science* ***275***: 218-220, (1997)). A similar effect is also found in substances such as catechin, epicatechin-3-gallate, epigallocatechin and epigallocatechin-3-gallate, which are found in the leaves of tea (*Camellia sinensis*). Beverages, in particular those made with unfermented tea leaves (green tea), are beneficial for health (Hu, G.; Han, C.; Chen, J. Inhibition of oncogene expression by green tea and (-)-epigallocatechin gallate in mice. *Nutr Cancer* **24:** 203-209; (1995); Scholz, E; Bertram, B. *Camellia sinensis* (L.) O. Kuntze. Der Teestrauch [the tea shrub]. *Z. Phytotherapie* ***17: 235-250,*** (1995); Yu, R.; Jiao, J. J.; Duh, J. L.; Gudehithlu, K.; Tan, T. H.; Kong, A. N. Activation of mitogen-activated protein kinases by green tea polyphenols: potential signaling pathways in the regulation of antioxidant responsive elements-mediated phase II enzyme gene expression. *Carcinigenesis* **18:** 451-456, (1997); Jankun, J.; Selman, S.H.; Swiercz, R. Why drinking green tea could prevent cancer. *Nature* ***387:*** *561,* (1997)). In addition,

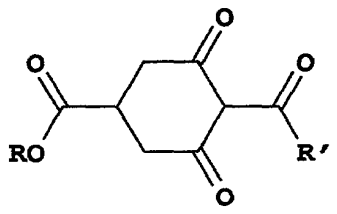
polymethoxylated flavones from citrus fruits also have a potential antitumor action (Chem, J.; Montanari, A.M.; Widmer, W.W. Two new polymethoxylated flavones, a class of compounds with potential anticancer activity, isolated from cold pressed dancy tangerine peel oil solids. *J Agric Food Chem* ***45:*** *364-368,* (1997)).

Acylcyclohexanediones such as prohexadione-calcium and trinexapac-ethyl (earlier name: cimectacarb) are employed as bioregulators for inhibiting longitudinal growth in plants. Their bioregulatory action is based on their blockage of the biosynthesis of gibberellins, which promote longitudinal growth. Owing to their structural relationship with 2-oxoglutaric acid, they inhibit certain dioxygenases which require 2-oxoglutaric acid as co-substrate (Rademacher, W, Biochemical effects of plant growth retardants, in: Plant Biochemical Regulators, Gausman, HW (ed.), Marcel Dekker, Inc., New York, pp. 169-200 (1991)). It is known that such compounds also engage in the phenol metabolism and can therefore cause inhibition of anthocyanin production in various kinds of plants (Rademacher, W et al., The mode of action of acylcyclohexanediones - a new type of growth retardant, in: Progress in Plant Growth Regulation, Karssen, CM, van Loon, LC, Vreugdenhil, D (eds.), Kluwer Academic Publishers, Dordrecht (1992)). Such effects on the balance of phenolic constituents are given as the cause of the side effect of prohexadione-calcium against fire blight (Rademacher, W et al., prohexadione-Ca - a new plant growth regulator for apple with interesting biochemical features, Poster presented at the 25th Annual Meeting of the Plant Growth Regulation Society of America, July 7-10, 1998, Chicago). A. Lux-Endrich (PhD thesis at the Technical University Munich at Weihenstephan, 1998) found during her studies into the mechanism of action of prohexadione-calcium against fire blight that, in apple tissue cultures, prohexadione-calcium results in the content of phenolic substances being increased several times and that a series of phenols is found which is otherwise not present. It was also found during this study that exposure to prohexadione-calcium leads to relatively large amounts of luteoliflavan and eriodictyol in the shoot tissue of apples. Luteoliflavan does normally not occur in apple tissue, and eriodictyol occurs only in small amounts as an intermediate in the flavonoid metabolism. However, the expected flavonoids catechin and cyanidin were not detectable in the treated tissue, or found in considerably reduced amounts only (S. Römmelt et al., paper presented at the 8th International Workshop on Fire Blight, Kusadasi, Turkey, October 12-15, 1998).

It can be considered as proven that prohexadione-calcium, trinexapac-ethyl and other acylcyclohexanediones inhibit 2-oxoglutaric-acid-dependent hydroxylases which are of importance in the metabolism of phenolic substances. These hydroxylases are primarily chalcone synthetase (CHS) and flavanone 3-hydroxylase (F3H) (W. Heller and G. Forkmann, Biosynthesis, in: The Flavonoids, Harborne, JB (ed.), Chapman and Hall, New York, 1988). However, it cannot be excluded that acylcyclohexanediones also inhibit other 2-oxoglutaric-acid-dependent hydroxylases which are as yet unknown. Furthermore, it should be obvious that lack of catechin, cyanidin or other end products of flavonoid synthesis is registered by the plant and that the activity of the key enzyme phenylalanine ammonium-lyase (PAL) is increased by a feedback mechanism. However, since CHS and F3H are still being inhibited, these flavonoid end products cannot be formed, and the result is an increased production of luteoliflavan, eriodictyol and other phenols (Figure 1).

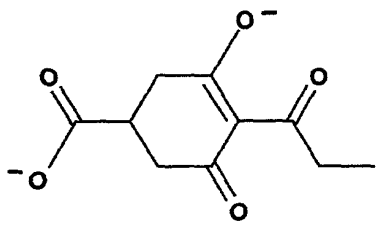
It is an object of the present invention to provide an economic, simple method for increasing the content of flavonoids and phenolic compounds in plants and to improve their health-promoting properties.

We have found that this object is achieved, surprisingly, by treating the plants with the growth-regulating compounds from the group of the acylcyclohexanediones(I)



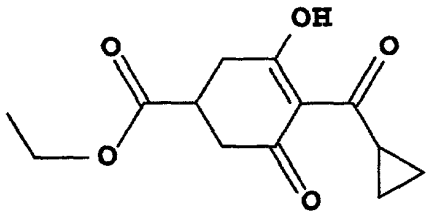
(I)

in particular with the compounds prohexadione-calcium (II)



Ca^{++} (II)

and trihexapac-ethyl (III)



(III).

Treatment of the plants with the acylcyclohexanediones of the formula (I), prohexadione-calcium (II) and trinexapac-ethyl (III) allows the flavonoids eriodictyol, proanthocyanidines, which are substituted on the C-atom 3 by hydrogen, for example luteoforol, luteoliflavan, apigeniflavan and tricetiflavan, and homogeneous and heterogeneous oligomers and polymers of the abovementioned, structurally related, substances to be formed in greater quantities.

Increased concentrations of the phenols hydroxycinnamic acid (p-coumaric acid, ferulic acid, sinapic acid,) salicylic acid or umbelliferone, including the homogeneous and heterogeneous oligomers and polymers formed with them, can be identified after the compounds acylcyclohexanedione of the formula (I), prohexadione-calcium (II) and trihexapac-ethyl (III) have been applied to plants.

The concentration of the glycosides of the flavonoids, of the phenolic compounds, of the chalcones and of the stilbenes in the plants is also increased by treating the plants with the acylcyclohexanediones of the formula (I), prohexadione-calcium (II) and trinexapac-ethyl (III).

Also, prohexadione-calcium, trinexapac-ethyl and related compounds engage in other metabolic reactions where, as yet, it has only been possible to assume that 2-oxoglutarate-dependent dioxygenases are involved.

A further additional positive effect when obtaining preparations from higher plants with an improved curative, health-promoting or tonifying action is that, owing to the growth-regulatory action of prohexadione-calcium, trinexapac-ethyl or related acylcyclohexanediones, a concentration effect of the relevant constituents results in the biological material.

The method according to the invention for increasing the content of flavonoids and phenolic constituents by treating the plants with compounds from the group of the acylcyclohexanediones of the

formula I, specifically prohexadione-calcium or trinexapac-ethyl, can be applied successfully to the following plants, but it is also possible successfully to treat plants which are not mentioned: grapevines, cherries, plums, sloes, blueberries, strawberries, citrus fruit (such as oranges, grapefruit), pawpaw, red cabbage, broccoli, Brussel sprouts, kale, carrots, parsley, celery/celeriac, onions, garlic, tea, coffee, cacao, maté, hops, soya, oilseed rape, oats, wheat, rye, Aronia melanocarpa and Ginkgo biloba.

Plants which have been treated with compounds from the group of the acylcyclohexanediones, specifically prohexadione-calcium or trihexapac-ethyl, in order to increase the content of flavonoids and phenolic compounds, or parts of these plants or products prepared from them (juices, infusions, extracts, fermentation products and fermentation residues) can be used for preparation of curative compositions, health-promoting compositions or tonics for humans and animals, and of cosmetics.

It is also possible to prepare, from the plants which have been treated in accordance with the invention, compositions wherein grapes of red grapevines are harvested and processed whose anthocyanin production has been prevented fully or partially by treatment with acylcyclohexanediones such as prohexadione-calcium or trinexapac-ethyl and which are therefore distinguished by a qualitatively and quantitatively increased content of flavonoids and other phenolic constituents.

Surprisingly, it has been found that the effect of a treatment with acylcyclohexanediones of the formula I, prohexadione-Ca or trihexapac-ethyl, causes the following to be observed in the plants, parts of these plants or products prepared from them (infusions, extracts, fermentation products, juices and the like):

(1) the antioxidative capacity *in vitro* (electron spin resonance (ESR), LDL oxidation, total antioxidant capacity, NO scavenging) is improved;

(2) a modulating effect on enzymes, especially signal transduction enzymes (protein kinase C, tyrosin protein kinase, phosphatidylinositol 3-kinase) is observed;

(3) a modulation of redox-sensitive transcriptional factors (NF-kB, AP-1) in endothelial cells, lymphocytes and smooth muscle cells is induced;

(4) the regulation of gene expression of target genes which are involved in the pathogenesis of inflammatory diseases (cytokines IL-1 and IL-8, *macrophage chemoattractant protein* 1 (MCP-1), adhesion factors ICAM-1 and VCAM-1) is modulated;

(5) an antiaggregatory action is induced;

(6) the cholesterol synthesis in the hepatocytes is inhibited;

(7) antiproliferative/antineoplastic effects are observed.

Example 1

Increase of the eriodictyol and luteoliflavan content in young apple leaves following treatment with prohexadione-calcium.

Apple plants cv. "Weirouge" were grown under controlled-environment conditions and treated to runoff point with 250 ppm prohexadione-calcium (formulated as BAS 125 10 W = wettable granules, content 10%). At various points in time after the treatment, the youngest fully developed leaf was harvested from each individual shoot. The freeze-dried leaves which had been ground using a pestle and mortar were extracted with methanol. Flavonoids and related compounds in the concentrated extract were analyzed by HPLC. Separation was performed on Hypersil ODS (particle size 3 µm) on a 250 x 4 mm column. Elution was carried out at a flow rate of 0.5 ml per minute, and mixtures of formic acid (5% in water) and methanol, increased stepwise from a ratio of 95 : 5 to 10 : 90 (v/v) were used. Phenolic acids and flavonols were detected at 280 nm. Flavan-3-ols were determined by post-column derivatization with p-dimethylaminocinnamaldehyde at 640 nm. For methodological details, see Treutter et al. (1994), Journal of Chromatography A 667, 290 - 297.

The result is shown in the table which follows:

Leaves treated with prohexadione-calcium show a markedly increased eriodictyol concentration after 12 and 21 days.

Treatment	Eriodictyol [g/kg dry matter]		Luteoliflavan [g/kg dry matter]	
	12 days after treatment	21 days after treatment	12 days after treatment	21 days after treatment.
Control	0	1	0	70
250 ppm prohexadione-calcium	17	27	0	34

Example 2

Preparation of sample materials from treated and untreated Dornfelder grapes

Vines cv. "Dornfelder" were treated twice at different points in time with the formulation BAS 125 10W, which contains prohexadione-calcium. 1000 g of prohexadione-calcium in 1000 l of spray mixture were applied per ha per treatment.

The 1st application was carried out at developmental stage 73 before the berries developed their color, and the 2nd application 10 days thereafter.

When harvested, the untreated and treated grapes showed a similar degree of ripeness. Untreated control: 69° Oechsle, acid: 7.3 g/l; treated control: 67° Oechsle, acid: 7.4 g/l.

Pigmentation was less pronounced in the treated grapes. As regards taste, no difference was observed.

The grapes were made into red wine by customary methods, i.e. the must was left to stand on the pulp for a prolonged period to improve pigment extraction.

After the wine which was free from cloudiness had been freeze-dried, approx. 2.5 g of a syrupy residue was obtained from 100 ml of untreated wine and approx. 2.1 g of syrupy residue from the wine from those vines which had been treated with prohexadione-calcium.

Example 3

Inhibition of cholesterol biosynthesis in primary rat hepatocyte cultures by prohexadione-calcium treated Dornfelder wine.

Preparation of the stock solutions

A quantity of the lyophilisate of the untreated and treated Dornfelder wines of between 10 and 20 mg was weighed exactly and treated with such an amount of DMSO that a stock solution of 10 mM total flavonoids resulted. These stock solutions were used for preparing dilutions in the culture medium immediately prior to the beginning of the test. The dilutions were done in 10-fold dilution steps of between 10^{-4} and 10^{-8} M.

Preparation of the hepatocyte cultures

Primary hepatocytes were obtained from the livers of Sprague-Dawley rats (240-290 g) by means of collagenase perfusion (Gebhardt et al., Arzneimittel-Forschung/Drug Res. 41: 800 - 804 (1991) 1990). They were cultured in collagen-coated Petri dishes (6-well plates, Greiner, Nürtingen) at a cell density of 125,000 cells/cm^2 in Williams medium E supplemented with 10% calf serum. More detailed information, in particular on the culture medium, are found in Gebhardt et al., Cell Biol. Toxicol. 6: 369 - 372 (1990) and Mewes et al., Cancer Res. 53: 5135 - 5142 (1993). After 2 h, the cultures were transferred to serum-free medium supplemented with 0.1 µM insulin. After a further 20 h, they were employed in the experiments. The test substances were each tested in three independent cultures of 2-3 rats.

Incubation of the liver cell cultures with the test substances

To demonstrate that cholesterol biosynthesis is influenced by the test substances, the hepatocyte cultures were maintained for 22 h in total. Then, they were incubated for 2 h in serum-free Williams medium E supplemented with ^{14}C-acetate (tracer quantities only) with the test substances at the concentrations indicated. Each test series included a control. The methodology is described in detail by Gebhardt (1991) and Gebhardt, Lipids 28: 613 - 619 (1993). The tracer quantities of ^{14}C-acetate exchange rapidly with the intracellular acetyl-CoA pool and therefore allow the incorporation of ^{14}C-acetate into the sterol fraction, > 90% of which consists of cholesterol, to be detected without interference (Gebhardt, 1993).

Analysis of the effect on cholesterol biosynthesis

The incorporation of ^{14}C-acetate into the sterol fraction (non-hydrolyzable lipids) was measured using the method of Gebhardt (1991). If the extraction is carried out by means of Extrelut® columns (Merck, Darmstadt), over 95% of the ^{14}C-acetate (and other low-molecular-weight metabolites formed therefrom in minor quantities) is removed. This test can provide comparative information on the relative synthesis rate of cholesterol and precursor sterols under the influence of test substances (Gebhardt, 1993).

Visual and microbial quality checks of the hepatocyte cultures

Before and after the test incubation, all cultures used were checked visually under the microscope for contamination with microorganisms and for the integrity of the cell monolayer. In none of the samples was a noticeable change in cell morphology observed (in particular at the higher concentrations). This largely rules out the possiblity that the test results were influenced by cytotoxic effects of the test substances.

The sterility tests, which were carried out routinely in all cultures, did not suggest any contamination with microorganisms.

Results

The untreated Dornfelder wine showed no effects whatsoever on cholesterol biosynthesis. In contrast, the cholesterol synthesis was inhibited significantly by samples of wine which originated from prohexadione-calcium-treated vines. At a concentration of 10^{-5} M, the inhibitory effect was approx. 60% and at 10^{-4} M almost 100%.

Example 4

Effect of prohexadione-Ca-treated wine extract (P-Ca) on the destruction of tumor cells

Confluent murine leukemia cells (RAW 264.7) and normal macrophages from the peritoneum of rats were cultivated in DMEM medium supplemented with fetal calf serum. Extracts of untreated and prohexadione-Ca-treted wine were added to the culture medium, up to a dosage of 200 µg/ml. In parallel experiments, 10, 25 and 50 µg/ml wine extract were incubated together with 100 µM H_2O_2.

Wine extract of wine which had been treated with prohexadione-Ca *per se* up to a dosage of 200 µg/ml had no cytotoxic effect on the cell cultures examined. However, after addition of H_2O_2 prohexadione-Ca-treated extract increased cell death of the tumor cells (RAW 264.7) in a dose-dependent manner. This is documented in Figure 2 by the increase of the cytosolic enzyme lactate dehydrogenase (LDH) in the culture medium. In nontransformed macrophages, there was no increase of the cytotoxic effect of H_2O_2. In the tumor cell line, there was an accumulation of the protein from the tumor suppressor gene p53 in the cytoplasm, see Figure 3.

Prohexadione-treated wine extract increases H_2O_2-induced cytotoxicity of leukemia cells but is ineffective in normal macrophages. This tumor-cell-specific effect is also observed in the case of cytostatics which act via increased oxidative stress (for example anthracyclines). The mechanism of prohexadione-Ca-treated wine extract is p53-dependent.

Example 5

Effect of prohexadione-Ca-treated wine extract on NF-κB activation in endothelial cells

The experiment was carried out using cocultures of macrophages (RAW 264.7) and endothelial cells (ECV 304). The culture medium of the endothelial cells was admixed with human LDL (low-density lipoproteins) and resting or interferon-γ (IFN-γ)-activated (10 U/ml) macrophages. After 16 h of incubation, the nuclear protein fraction was separated off and DNA binding (activation) of the redox-sensitive transcription factor NF-κB was determined in an electrophoretic mobility shift assay.

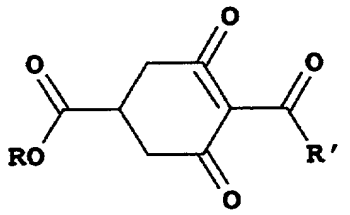
The basal content in the resting endothelial cells was typically low, see Figure 4. Addition of LDL resulted in activation of NF-κB, which was higher in activated macrophages than in resting macrophages. This corresponds to a physiological oxidation of LDL during atherogenesis. In all cases, incubation with prohexadione-Ca-treated wine extract resulted in an increased NF-κB activation.

The cell culture model used is highly suitable for describing the pathophysiological/inflammatory conditions in the early phase of atherosclerosis. NF-κB activation is enhanced by the prohexadione-Ca-treated wine extract. This is equivalent to the

action of a biological response modifier; i.e. the cellular response to a pathophysiological signal is enhanced in the positive sense.

New patent claims:

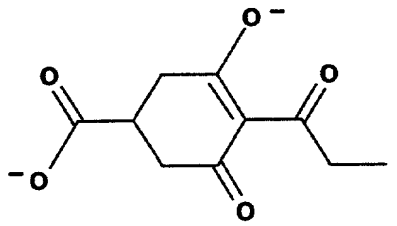
1. A method of increasing and qualitatively modifying the content of flavonoids and phenolic constituents in grapevines, cherries, plums, sloes, blueberries, strawberries, citrus fruit, pawpaw, red cabbage, broccoli, Brussels sprouts, kale, carrots, parsley, celery/celeriac, onions, garlic, tea, coffee, cacao, maté, hops, soya, oilseed rape, oats, wheat, rye, *Aronia melanocarpa* or *Ginkgo biloba*, which comprises treating the plants with an acylcyclohexadione [sic] of the formula I



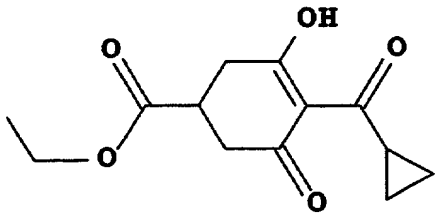
I,

where R is hydrogen or C_1-C_6-alkyl and R' is C_1-C_6-alkyl or C_3-C_6-cycloalkyl, or with a suitable salt of I.

2. A method as claimed in claim 1, wherein the plants are treated with an acylcyclohexadione [sic] of the formula II and/or the formula III



Ca^{++} II



III.

3. A method as claimed in claim 1 or 2, wherein the content of flavonoids and phenolic constituents of grapevines is increased and qualitatively modified.

4. A method as claimed in any of claims 1, 2 or 3, wherein the content of flavonoids with an unsubstituted C atom in the 3-position, and of the oligomers and polymers of these flavonoids, is increased.

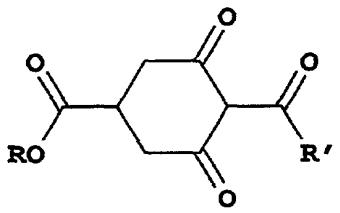
5. The use of grapevines, cherries, plums, sloes, blueberries, strawberries, citrus fruit, pawpaw, red cabbage, broccoli, Brussels sprouts, kale, carrots, parsley, celery/celeriac, onions, garlic, tea, coffee, cacao, maté, hops, soya, oilseed rape, oats, wheat, rye, *Aronia melanocarpa* or *Ginkgo biloba*, which have been treated with an acylcyclohexadione [sic] as set forth in claim 1 or 2, of parts of these plants or of products prepared with these plants (juices, teas, extracts, fermentation products and fermentation residues) for the preparation of curative compositions, health-promoting compositions or tonics for humans and animals, and of cosmetics.

6. An extract, juice, wine or press cake with an increased qualtitatively modified content of flavonoids and other phenolic constituents, obtainable from grapes of a red grapevine variety, the grapevine plant previously having been treated with at least one acylcyclohexadione [sic] of the formula I, II or III as set forth in claim 1 or 2.

Method of generating plants with an increased content of flavonoids and phenolic constituents

Abstract

In a method for increasing the flavonoid content in plants, the plants are treated with growth-regulating acylcyclohexanediones of the formula I.



(I)

Figure 1

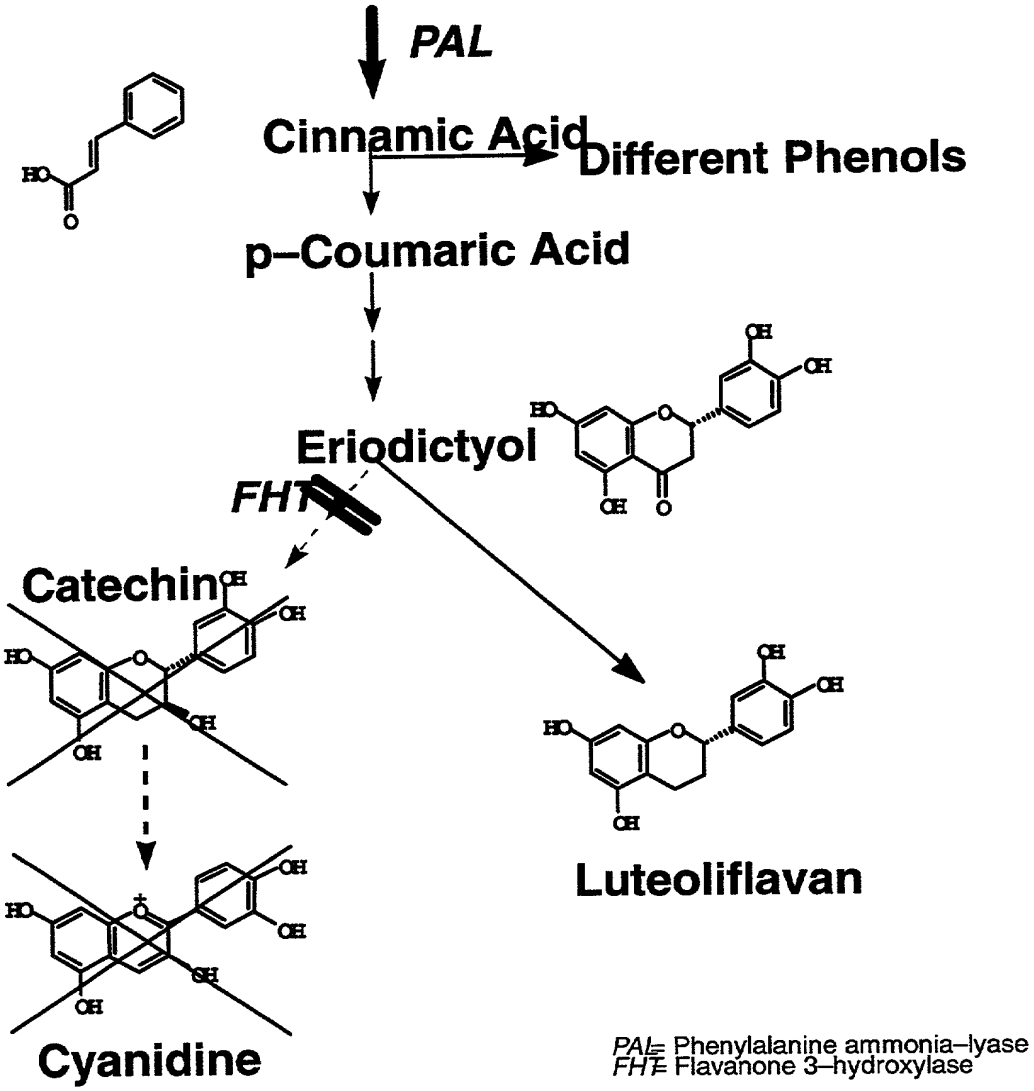


Figure 2

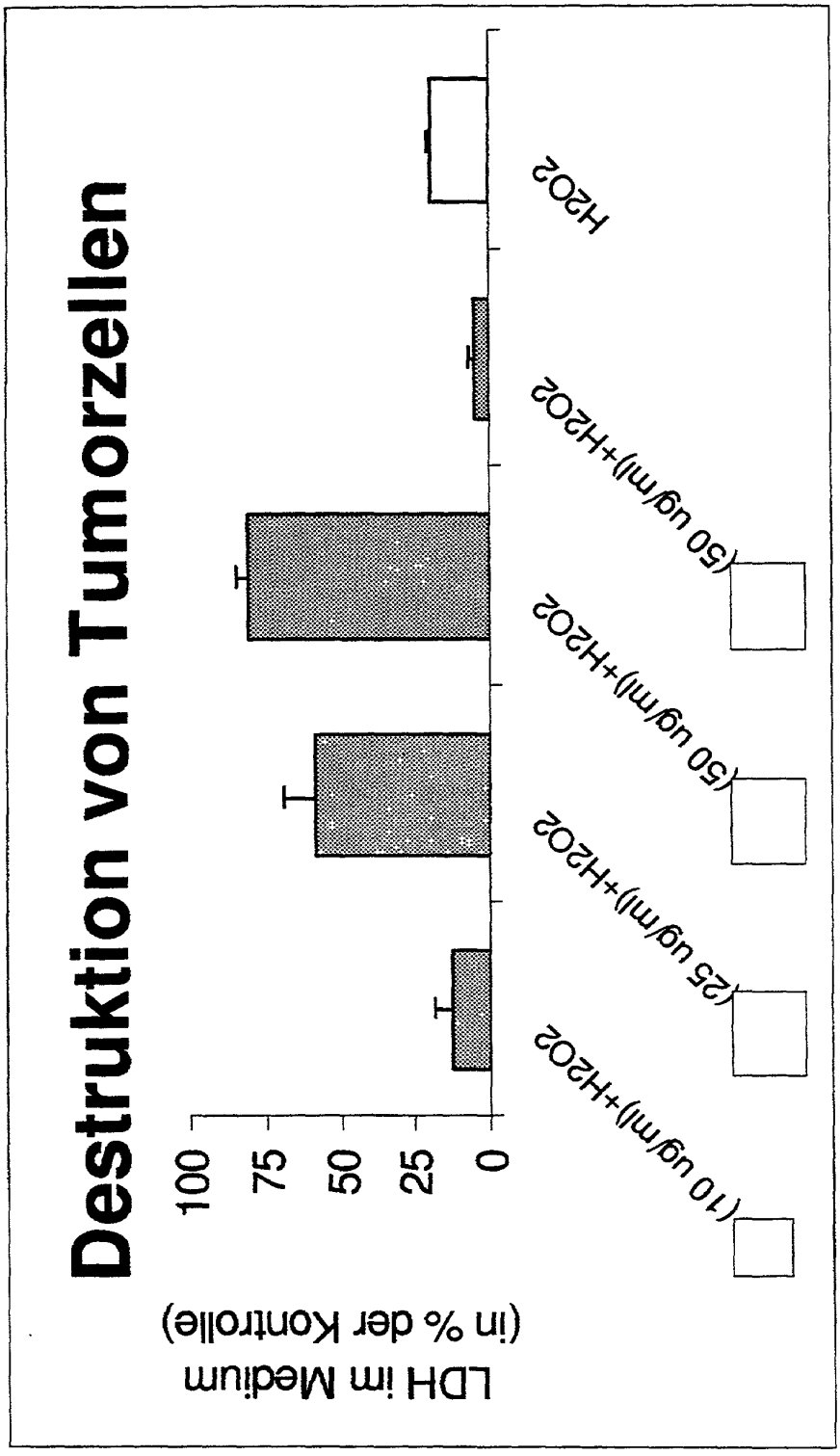

Destruktion von Tumorzellen
LDH im Medium
(in % der Kontrolle)
100
75
50
25
0
H2O2
(50 ug/ml)+H2O2
(50 ug/ml)+H2O2
(25 ug/ml)+H2O2
(10 ug/ml)+H2O2

Figure 3

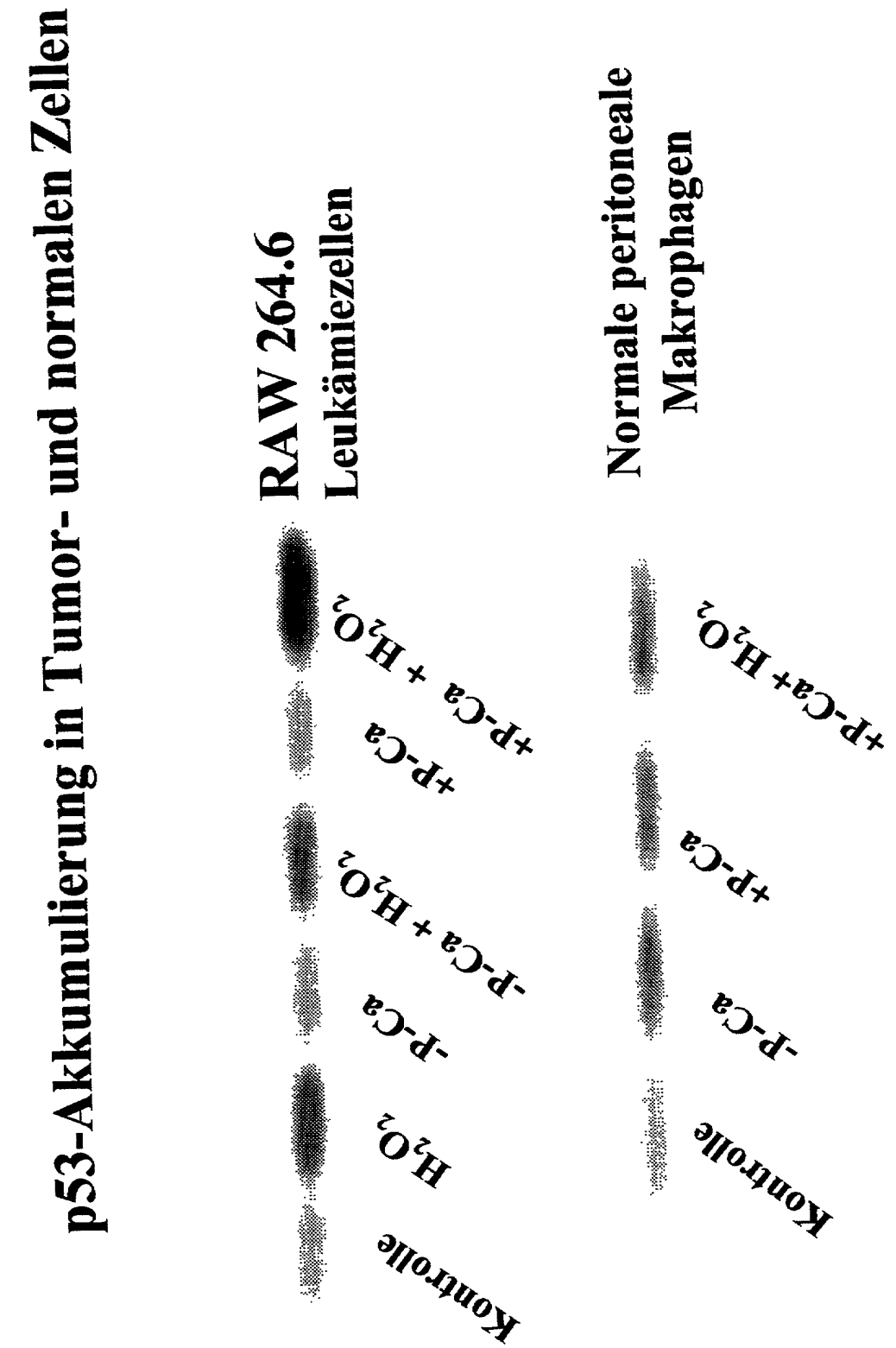
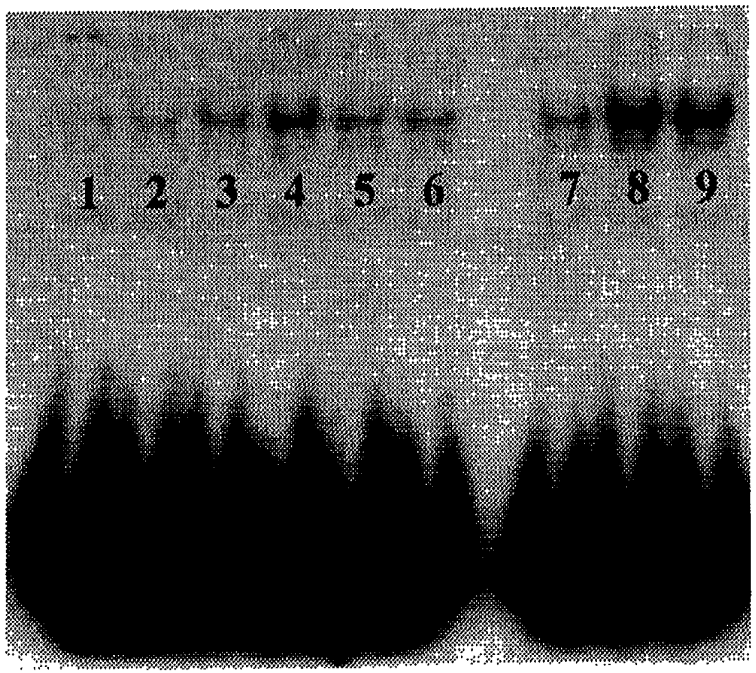

p53-Akkumulierung in Tumor- und normalen Zellen
RAW 264.6
Leukämiezellen
Kontrolle
H2O2
-p-Ca
-p-Ca + H2O2
+p-Ca
+p-Ca + H2O2
Normale peritoneale
Makrophagen
Kontrolle
-p-Ca
+p-Ca
+p-Ca+ H2O2

Figure 4

NF-κB -Aktivierung in Endothelzellen



1 = Endothelzellen (EC)
2 = EC + IFN-γ
3 = EC + ruhende Makrophagen (RM)
4 = EC + aktivierte Makrophagen (AM)
5 = EC + LDL + RM
6 = EC + LDL + AM

7 = EC + P-Ca (25 μg/ml) + LDL + RM
8 = EC + P-Ca (25 μg/ml) + AM
9 = EC + P-Ca (25 μg/ml) + LDL + AM

Declaration, Power of Attorney

0050/050061

We (I), the undersigned inventor(s), hereby declare(s) that:

My residence, post office address and citizenship are as stated below next to my name,

We (I) believe that we are (I am) the original, first, and joint (sole) inventor(s) of the subject matter which is claimed and for which a patent is sought on the invention entitled

Method of generating plants with an increased content of flavonoids and phenolic constituents

the specification of which

[X] is attached hereto.

[ ] was filed on _____ as

Application Serial No. _____

and amended on _____.

[x] was filed as PCT international application

Number PCT/EP00/05258

on June 7, 2000

and was amended under PCT Article 19

on _____ (if applicable).

We (I) hereby state that we (I) have reviewed and understand the contents of the above–identified specification, including the claims, as amended by any amendment referred to above.

We (I) acknowledge the duty to disclose information known to be material to the patentability of this application as defined in Section 1.56 of Title 37 Code of Federal Regulations.

We (I) hereby claim foreign priority benefits under 35 U.S.C. § 119(a)–(d) or § 365(b) of any foreign application(s) for patent or inventor's certificate, or § 365(a) of any PCT International application which designated at least one country other than the United States, listed below and have also identified below, by checking the box, any foreign application for patent or inventor's certificate, or PCT International application having a filing date before that of the application on which priority is claimed. Prior Foreign Application(s)

Application No.	**Country**	**Day/Month/Year**	**Priority Claimed**
19927571.8	*Germany*	*17 June 1999*	[x] Yes [ ] No

We (I) hereby claim the benefit under Title 35, United States Codes, § 119(e) of any United States provisional application(s) listed below.

(Application Number)	(Filing Date)
(Application Number)	(Filing Date)

We (I) hereby claim the benefit under 35 U.S.C. § 120 of any United States application(s), or § 365(c) of any PCT International application designating the United States, listed below and, insofar as the subject matter of each of the claims of this application is not disclosed in the prior United States or PCT International application in the manner provided by the first paragraph of 35 U.S.C. § 112, I acknowledge the duty to disclose information which is material to patentability as defined in 37 CFR § 1.56 which became available between the filing date of the prior application and the national or PCT International filing date of this application.

Application Serial No.	**Filing Date**	**Status (pending, patented, abandoned)**

And we (I) hereby appoint **Messrs. HERBERT. B. KEIL**, Registration Number 18,967; and **RUSSEL E. WEINKAUF**, Registration Number 18,495; the address of both being Messrs. Keil & Weinkauf, 1101 Connecticut Ave., N.W., Washington, D.C. 20036 (telephone 202–659–0100), our attorneys, with full power of substitution and revocation, to prosecute this application, to make alterations and amendments therein, to sign the drawings, to receive the patent, and to transact all business in the Patent Office connected therewith.

We (I) declare that all statements made herein of our (my) own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issuing thereon.

1-10

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